APPENDIX

Exhibits A through J

APPENDIX

EXHIBIT A

EXHIBIT A

USPTO Patent Full-Text and Image Database

Searching US Patent Collection...

Results of Search in US Patent Collection db for:
ACLM/"cleavable linker" AND ACLM/protease: 23 patents.
Hits 1 through 23 out of 23

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Refine Search ACLM/"cleavable linker" AND ACLM/protease

	PAT. NO.	Title
1	7,511,124	Compositions comprising phosphatidylethanolamine-binding peptides linked to anti-viral agents
2	7,470,661	Delivery of superoxide dismutase to neuronal cells
3	7,455,833	Methods and compositions for treating viral infections using antibodies and immunoconjugates to aminophospholipids
4	7,422,865	Method of identifying peptides in a proteomic sample
5	7,384,909	Anti-viral treatment methods using phosphatidylethanolamine-binding peptides linked to anti-viral agents
6	7,279,564	Expression in filamentous fungi of protease inhibitors and variants thereof
7	7,232,805	Cobalamin conjugates for anti-tumor therapy
8	7,138,381	Compositions containing nucleic acids and ligands for therapeutic treatment
9	7,132,519	Releasable nonvolatile mass-label molecules
10	7,063,847	Compositions and methods for enhancing immune responses mediated by antigen-presenting cells
11	6,888,047	Transgenic animals as urinary bioreactors for the production of polypeptide in the urine, recombinant DNA construct for kidney-specific expression, and method of using same
12	6,676,941	Antibody conjugate formulations for selectively inhibiting VEGF
13	6,590,078	DNA sequences, vectors, and fusion polypeptides for secretion of polypeptides in filamentous fungi
14	6,586,411	System for monitoring the location of transgenes
15	6,503,886	Compositions containing nucleic acids and ligands for therapeutic treatment
16	6,416,758	Antibody conjugate kits for selectively inhibiting VEGF
17	6,342,221	Antibody conjugate compositions for selectively inhibiting VEGF

18 6,265,204 T DNA sequences, vectors, and fusion polypeptides for secretion of polypeptides in filamentous fungi

19 6,130,063 T DNA sequences, vectors, and fusion polypeptides to increase secretion of desired polypeptides from filamentous fungi

20 6,037,329 T Compositions containing nucleic acids and ligands for therapeutic treatment

21 5,679,543 T DNA sequences, vectors and fusion polypeptides to increase secretion of desired polypeptides from filamentous fungi

22 5,654,176 T Fusion proteins containing glutathione-s-transferase

23 4,671,958 T Antibody conjugates for the delivery of compounds to target sites

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EXHIBIT B

EXHIBIT B

US007511124B2

(12) **United States Patent**
Thorpe et al.

(10) **Patent No.: US 7,511,124 B2**
(45) **Date of Patent: Mar. 31, 2009**

(54) **COMPOSITIONS COMPRISING PHOSPHATIDYLETHANOLAMINE-BINDING PEPTIDES LINKED TO ANTI-VIRAL AGENTS**

(75) Inventors: **Philip E. Thorpe**, Dallas, TX (US); **M. Melina Soares**, Richardson, TX (US); **Jin He**, Dallas, TX (US)

(73) Assignee: **Board of Regents, The University of Texas System**, Austin, TX (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 692 days.

(21) Appl. No.: **10/642,121**

(22) Filed: **Aug. 15, 2003**

(65) **Prior Publication Data**

US 2005/0059578 A1 Mar. 17, 2005

Related U.S. Application Data

(63) Continuation-in-part of application No. 10/621,269, filed on Jul. 15, 2003.

(60) Provisional application No. 60/396,263, filed on Jul. 15, 2002.

(51) **Int. Cl.**
C07K 14/00 (2006.01)
A61K 31/00 (2006.01)

(52) **U.S. Cl.** **530/402**; 514/2; 514/350

(58) **Field of Classification Search** 530/402; 514/2, 350

See application file for complete search history.

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(57) **ABSTRACT**

Disclosed are surprising discoveries concerning the role of anionic phospholipids and aminophospholipids in tumor vasculature and in viral entry and spread, and compositions and methods for utilizing these findings in the treatment of cancer and viral infections. Also disclosed are advantageous antibody, immunoconjugate and duramycin-based compositions and combinations that bind and inhibit anionic phospholipids and aminophospholipids, for use in the safe and effective treatment of cancer, viral infections and related diseases.

56 Claims, 53 Drawing Sheets

-continued

```
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What is claimed is:

1. A phosphatidylethanolamine binding peptide conjugate, comprising a phosphatidylethanolamine (PE) binding peptide (PE-binding peptide) operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a kininogen, a cinnamycin peptide or a duramycin peptide.

2. The PE-binding peptide conjugate of claim **1**, wherein said at least a first anti-viral agent is cidofovir.

3. The PE-binding peptide conjugate of claim **1**, wherein said PE-binding peptide is a cinnamycin peptide.

4. The PE-binding peptide conjugate of claim **1**, wherein said PE-binding peptide is a duramycin peptide.

5. The PE-binding peptide conjugate of claim **1**, wherein said at least a first anti-viral agent is a nucleoside.

6. The PE-binding peptide conjugate of claim **1**, wherein said at least a first anti-viral agent is a reverse transcriptase inhibitor.

7. The PE-binding peptide conjugate of claim **1**, wherein said at least a first anti-viral agent is a protease inhibitor.

8. The PE-binding peptide conjugate of claim **1**, wherein said at least a first anti-viral agent is acyclovir, penciclovir (famciclovir), gancyclovir (ganciclovir), deoxyguanosine, foscarnet, idoxuridine, trifluorothymidine, vidarabine, sorivudine, zidovudine, didanosine, zalcitabine, lamivudine, stavudine, abacavir, multinucleoside resistance A, multinucleoside resistance B, nevirapine, delavirdine, efavirenz, adefovir dipivoxil, indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, deoxycytosine triphosphate, lamivudine triphosphate, emticitabine triphosphate, adefovir diphosphate, penciclovir triphosphate, lobucavir triphosphate, amantadine, rimantadine, zanamivir or oseltamivir.

9. The PE-binding peptide conjugate of claim **1**, wherein said at least a first anti-viral agent is AZT.

10. The PE-binding peptide conjugate of claim **1**, wherein said PE-binding peptide is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

11. The PE-binding peptide conjugate of claim **1**, wherein said PE-binding peptide conjugate is comprised in a pharmaceutically acceptable composition.

12. The PE-binding peptide conjugate of claim 11, wherein said pharmaceutically acceptable composition is formulated for intravenous administration.

13. The PE-binding peptide conjugate of claim 11, wherein said pharmaceutically acceptable composition is formulated for administration as an aerosol.

14. The PE-binding peptide conjugate of claim 11, wherein said pharmaceutically acceptable composition further comprises at least a second anti-viral agent.

15. A composition comprising a biologically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a kininogen, a cinnamycin peptide or a duramycin peptide.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a kininogen, a cinnamycin peptide or a duramycin peptide.

17. The pharmaceutical composition of claim 16, wherein said composition further comprises at least a second anti-viral agent.

18. A kit comprising, in at least a first container, a combined effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to an anti-viral agent, and at least a second, distinct anti-viral agent; wherein said PE-binding peptide is a kininogen, a cinnamycin peptide or a duramycin peptide.

19. The kit of claim 18, wherein said at least a second, distinct anti-viral agent is a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

20. The kit of claim 18, wherein said PE-binding peptide conjugate and said at least a second, distinct anti-viral agent are comprised within a single container.

21. The kit of claim 18, wherein said PE-binding peptide conjugate and said at least a second, distinct anti-viral agent are comprised within distinct containers.

22. The PE-binding peptide conjugate of claim 1, wherein said PE-binding peptide is covalently linked to said at least a first anti-viral agent.

23. The PE-binding peptide conjugate of claim 1, wherein said PE-binding peptide is conjugated to said at least a first anti-viral agent.

24. The PE-binding peptide conjugate of claim 1, wherein said PE-binding peptide conjugate is a recombinant fusion protein.

25. The PE-binding peptide conjugate of claim 1, wherein said PE-binding peptide is operatively attached to said at least a first anti-viral agent via an avidin:biotin bridge.

26. The PE-binding peptide conjugate of claim 1, wherein said PE-binding peptide is operatively attached to said at least a first anti-viral agent via a cross-linker or peptide spacer.

27. The PE-binding peptide conjugate of claim 1, wherein said PE-binding peptide is a kininogen.

28. A phosphatidylethanolamine binding peptide conjugate, comprising a phosphatidylethanolamine (PE) binding peptide (PE-binding peptide) operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a kininogen.

29. The PE-binding peptide conjugate of claim 28, wherein said at least a first anti-viral agent is AZT or cidofovir.

30. The PE-binding peptide conjugate of claim 29, wherein said at least a first anti-viral agent is a nucleoside reverse transcriptase inhibitor.

31. The PE-binding peptide conjugate of claim 29, wherein said at least a first anti-viral agent is a non-nucleoside reverse transcriptase inhibitor.

32. The PE-binding peptide conjugate of claim 28, wherein said at least a first anti-viral agent is a protease inhibitor.

33. A composition comprising a biologically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a kininogen.

34. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a kininogen.

35. A kit comprising, in at least a first container, a combined effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to an anti-viral agent, and at least a second, distinct anti-viral agent; wherein said PE-binding peptide is a kininogen.

36. A phosphatidylethanolamine binding peptide conjugate, comprising a phosphatidylethanolamine (PE) binding peptide (PE-binding peptide) operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide.

37. The PE-binding peptide conjugate of claim 36, wherein said PE-binding peptide is a cinnamycin peptide.

38. The PE-binding peptide conjugate of claim 36, wherein said PE-binding peptide is a duramycin peptide.

39. The PE-binding peptide conjugate of claim 36, wherein said at least a first anti-viral agent is a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

40. A composition comprising a biologically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide.

41. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide.

42. A kit comprising, in at least a first container, a combined effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to an anti-viral agent, and at least a second, distinct anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide.

43. A phosphatidylethanolamine binding peptide conjugate, comprising a phosphatidylethanolamine (PE) binding peptide (PE-binding peptide) attached to at least a first anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide and wherein said PE-binding peptide is attached to said at least a first anti-viral agent via a covalent linkage, conjugation, preparation as a recombinant fusion protein, an avidin:biotin bridge, a cross-linker, peptide spacer, a biologically releasable bond or selectively cleavable linker.

44. The PE-binding peptide conjugate of claim 43, wherein said PE-binding peptide is a cinnamycin peptide.

45. The PE-binding peptide conjugate of claim 43, wherein said PE-binding peptide is a duramycin peptide.

46. The PE-binding peptide conjugate of claim 43, wherein said at least a first anti-viral agent is a nucleoside reverse

transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

47. A composition comprising a biologically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide and wherein said PE-binding peptide is attached to said at least a first anti-viral agent via a covalent linkage, conjugation, preparation as a recombinant fusion protein, an avidin:biotin bridge, a cross-linker, peptide spacer, a biologically releasable bond or selectively cleavable linker.

48. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to at least a first anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide and wherein said PE-binding peptide is attached to said at least a first anti-viral agent via a covalent linkage, conjugation, preparation as a recombinant fusion protein, an avidin:biotin bridge, a cross-linker, peptide spacer, a biologically releasable bond or selectively cleavable linker.

49. A kit comprising, in at least a first container, a combined effective amount of a PE-binding peptide conjugate that comprises a PE-binding peptide operatively attached to an anti-viral agent, and at least a second, distinct anti-viral agent; wherein said PE-binding peptide is a cinnamycin peptide or a duramycin peptide and wherein said PE-binding peptide is attached to said at least a first anti-viral agent via a covalent linkage, conjugation, preparation as a recombinant fusion protein, an avidin:biotin bridge, a cross-linker, peptide spacer, a biologically releasable bond or selectively cleavable linker.

50. The PE-binding peptide conjugate of claim **36**, wherein said PE-binding peptide conjugate is comprised in a pharmaceutically acceptable composition.

51. The PE-binding peptide conjugate of claim **50**, wherein said pharmaceutically acceptable composition is formulated for intravenous administration.

52. The PE-binding peptide conjugate of claim **50**, wherein said pharmaceutically acceptable composition is formulated for administration as an aerosol.

53. The PE-binding peptide conjugate of claim **50**, wherein said pharmaceutically acceptable composition further comprises at least a second anti-viral agent.

54. The pharmaceutical composition of claim **41**, wherein said composition further comprises at least a second anti-viral agent.

55. The kit of claim **42**, wherein said PE-binding peptide conjugate and said at least a second, distinct anti-viral agent are comprised within a single container.

56. The kit of claim **42**, wherein said PE-binding peptide conjugate and said at least a second, distinct anti-viral agent are comprised within distinct containers.

* * * * *

EXHIBIT C

EXHIBIT C

US007470661B2

(12) **United States Patent**
Shone et al.

(10) **Patent No.: US 7,470,661 B2**
(45) **Date of Patent: Dec. 30, 2008**

(54) **DELIVERY OF SUPEROXIDE DISMUTASE TO NEURONAL CELLS**

(75) Inventors: **Clifford Charles Shone**, Salisbury (GB); **John Mark Sutton**, Salisbury (GB); **Bassam Hallis**, Salisbury (GB); **Nigel Silman**, Salisbury (GB)

(73) Assignee: **Syntaxin Limited**, Salisbury, Wiltshire (GB)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 373 days.

(21) Appl. No.: **11/062,471**

(22) Filed: **Feb. 22, 2005**

(65) **Prior Publication Data**

US 2005/0255093 A1 Nov. 17, 2005

Related U.S. Application Data

(63) Continuation of application No. 09/831,050, filed as application No. PCT/GB99/03699 on Nov. 5, 1999, now abandoned.

(30) **Foreign Application Priority Data**

Nov. 5, 1998 (GB) 9824282.9

(51) **Int. Cl.**

A61K 38/00	(2006.01)
A61K 38/16	(2006.01)
A61K 38/43	(2006.01)
C07K 14/00	(2006.01)
C12N 9/00	(2006.01)

(52) **U.S. Cl.** **514/2**; 514/6; 424/94.1; 530/350; 530/402; 435/183

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(Continued)

Primary Examiner—Elizabeth C. Kemmerer
Assistant Examiner—Sandra Wegert
(74) *Attorney, Agent, or Firm*—Sterne, Kessler, Goldstein & Fox PLLC

(57) **ABSTRACT**

A composition for delivery of superoxide dismutase to neuronal cells comprise a superoxide dismutase linked by a linker to a neuronal cell targeting component, which component comprises a first domain that binds to a neuronal cell and a second domain that translocates the superoxide dimutase into the neuronal cell. After translocation, the linker is cleaved to release superoxide dimutase from the neuronal cell targeting domain. Also described is use of the composition for treatment of oxidative damage to neuronal cells and further targeting of the composition using human mitochondrial leader sequences. A hybrid polypeptide is described that contains a bacterial superoxide dismutase plus a sequence that targets a human mitochondira.

5 Claims, 4 Drawing Sheets

-continued

```
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```

The invention claimed is:

1. A conjugate for delivery of superoxide dismutase (SOD) to a mitochondrion in a neuronal cell, comprising:

(i) SOD and a leader sequence, wherein said leader sequence targets the SOD to said mitochondrion; and

(ii) a neuronal cell targeting component,

wherein said neuronal cell targeting component is linked to the SOD by a cleavable linker selected from the group consisting of (a) a disulfide bridge, and (b) a site for a protease found in neuronal cells,

and wherein said neuronal targeting component comprises a first domain that binds to a neuronal cell, wherein said first domain comprises a clostridial neurotoxin cell binding domain, and a clostridial neurotoxin translocation domain that translocates the SOD into said neuronal cell.

2. The conjugate of claim 1, wherein the first domain comprises a H_C fragment of a clostridial neurotoxin H-chain.

3. The conjugate of claim 1, wherein the clostridial neurotoxin translocation domain comprises a H_N fragment of a clostridial neurotoxin H-chain.

4. The conjugate of claim 1, wherein the SOD is Mn-SOD.

5. The conjugate of claim 1, wherein the leader sequence that targets the SOD to the mitochondrion is derived from human Mn-SOD.

* * * * *

EXHIBIT D

EXHIBIT D

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Thorpe et al.

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(54) **METHODS AND COMPOSITIONS FOR TREATING VIRAL INFECTIONS USING ANTIBODIES AND IMMUNOCONJUGATES TO AMINOPHOSPHOLIPIDS**

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A61K 39/00 (2006.01)
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(52) **U.S. Cl.** **424/130.1**; 424/133.1; 424/141.1; 424/178.1; 424/183.1; 514/2

(58) **Field of Classification Search** 435/326, 435/337; 424/133.1, 141.1, 152.1, 172.1, 424/174.1, 277.1; 514/2; 530/387.1
See application file for complete search history.

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(57) **ABSTRACT**

Disclosed are surprising discoveries concerning the role of anionic phospholipids and aminophospholipids in tumor vasculature and in viral entry and spread, and compositions and methods for utilizing these findings in the treatment of cancer and viral infections. Also disclosed are advantageous antibody, immunoconjugate and duramycin-based compositions and combinations that bind and inhibit anionic phospholipids and aminophospholipids, for use in the safe and effective treatment of cancer, viral infections and related diseases.

118 Claims, 53 Drawing Sheets

-continued

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20

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<211> LENGTH: 15
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<210> SEQ ID NO 9
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<222> LOCATION: (11)..(18)
<223> OTHER INFORMATION: Xaa = Abu

<400> SEQUENCE: 9

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1 5 10 15

Asn Xaa Lys

What is claimed is:

1. A method of inhibiting virus replication or spread to additional host cells or tissues, comprising contacting a mammalian cell with an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins, in an amount effective to inhibit virus replication in said cell or to inhibit spread to additional host cells or tissues from said cell.

2. The method of claim 1, wherein said antibody is a monoclonal antibody or antigen-binding fragment thereof.

3. The method of claim 1, wherein said antibody is an IgG antibody or antigen-binding fragment thereof.

4. The method of claim 1, wherein said antibody is an antigen-binding fragment of an antibody.

5. The method of claim 4, wherein said antibody is an scFv, Fv, Fab', Fab, diabody, linear antibody, $F(ab')_2$ antigen-binding fragment of an antibody or a univalent fragment, camelized or single domain antibody.

6. The method of claim 1, wherein said antibody is a human, humanized or part-human antibody or an antigen-binding fragment thereof.

7. The method of claim 1, wherein said antibody is a chimeric, bispecific, recombinant or engineered antibody, or an antigen-binding fragment thereof.

8. The method of claim 1, wherein said antibody, or antigen-binding fragment thereof, binds to phosphatidylethanolamine.

9. The method of claim 1, wherein said antibody, or antigen-binding fragment thereof, binds to phosphatidylserine.

10. The method of claim 1, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

11. The method of claim 1, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

12. The method of claim 1, wherein said cell is a mouse cell.

13. The method of claim 1, wherein said cell is a human cell.

14. The method of claim 1, wherein said virus is a hepatitis, influenza, HIV, herpes, paramyxovirus, poxvirus, rhabdovirus or arenavirus.

15. The method of claim 1, wherein said method inhibits virus replication.

16. The method of claim 1, wherein said method inhibits virus spread to additional host cells or tissues.

17. The method of claim 1, wherein said cell is located within a mammal and said antibody, or antigen-binding fragment thereof is administered to said mammal.

18. The method of claim 17, wherein said mammal is a human patient.

19. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins, in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection.

20. The method of claim 19, wherein said pharmaceutical composition is administered to said mammal intravenously.

21. The method of claim 19, wherein said pharmaceutical composition is administered to said mammal as an aerosol.

22. The method of claim 19, wherein said mammal has, or is at risk for developing, hepatitis, influenza, AIDS, viral pneumonia or respiratory disease, smallpox, CMV mononucleosis or Lass a fever.

23. The method of claim 19, wherein at least a second, distinct anti-viral agent is administered to said mammal.

24. The method of claim 23, wherein said at least a second, distinct anti-viral agent is a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

25. The method of claim 19, wherein said mammal is a human patient.

26. The method of claim 23, wherein said at least a second, distinct anti-viral agent is cidofovir, acyclovir, penciclovir (famciclovir), gancyclovir (ganciclovir), deoxyguanosine, foscarnet, idoxuridine, trifluorothymidine, vidarabine, sorivudine, zidovudine, didanosine, zalcitabine, lamivudine, stavudine, abacavir, multinucleoside resistance A, multinucleoside resistance B, nevirapine, delavirdine, efavirenz, adefovir dipivoxil, indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, hydroxyurea, deoxycytosine triphosphate, lamivudine triphosphate, emticitabine triphosphate, adefovir diphosphate, penciclovir triphosphate, lobucavir triphosphate, interferon alpha, ribavirin, amantadine, rimantadine, zanamivir or oseltamivir.

27. The method of claim 19, wherein said

(d) viral infection is an infection with a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

28. The method of claim 27, wherein said viral infection is an infection with a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

29. A method of inhibiting virus replication or spread, comprising contacting a population of mammalian cells with an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins, in an amount effective to inhibit virus replication or spread in said population of cells.

30. The method of claim 29, wherein said population of cells is located within a mammal and said antibody, or antigen-binding fragment thereof is administered to said mammal.

31. The method of claim 30, wherein said mammal is a human patient.

32. The method of claim 29, wherein said

(d) virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

33. The method of claim 32, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

34. A method of inhibiting virus replication or spread, comprising contacting a population of mammalian cells with an antibody, or antigen-binding fragment thereof, in an amount effective to inhibit virus replication or spread in said population of cells; wherein said antibody, or antigen-binding fragment thereof, binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid.

35. The method of claim 34, wherein said virus is a virus from the Orthomyxoviridae, Poxviridae, Arenaviridae, Paramyxoviridae, Flaviviridae, Retroviridae, Rhabdoviridae or Herpesviridae family.

36. The method of claim 34, wherein said population of cells is located within a mammal and said antibody, or antigen-binding fragment thereof, is administered to said mammal.

37. The method of claim 36, wherein said mammal is a human patient.

38. The method of claim 34, wherein said

(d) wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

39. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising an antibody, or antigen-binding fragment thereof, in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection; wherein said antibody, or antigen-binding fragment thereof, binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid.

40. The method of claim 39, wherein said viral infection is an infection with a virus from the Orthomyxoviridae, Poxviridae, Arenaviridae, Paramyxoviridae, Flaviviridae, Retroviridae, Rhabdoviridae or Herpesviridae family.

41. The method of claim 39, wherein said mammal is a human patient.

42. The method of claim 39, wherein said

(d) viral infection is an infection with a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

43. A method of inhibiting virus replication or spread to additional host cells or tissues, comprising contacting a mammalian cell with an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins, in an amount effective to inhibit virus replication in said cell or to inhibit spread to additional host cells or tissues from said cell.

44. The method of claim 43, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

45. The method of claim 43, wherein said

(d) virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

46. A method of inhibiting virus replication or spread, comprising contacting a population of mammalian cells with an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins, in an amount effective to inhibit virus replication or spread in said population of cells.

47. The method of claim 46, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

48. The method of claim 46, wherein said

(d) virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

49. The method of claim 46, wherein said population of cells is located within a mammal and said immunoconjugate is administered to said mammal.

50. The method of claim 46, wherein said population of cells is located within a human patient and said immunoconjugate is administered to said human patient.

51. The method of claim 46, wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

52. The method of claim 46, wherein said antibody, or antigen-binding fragment thereof, is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

53. The method of claim 46, wherein said antibody is a human, humanized, part-human, chimeric, bispecific, recombinant or engineered antibody, or an antigen-binding fragment thereof.

54. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins, in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection.

55. The method of claim 54, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

56. The method of claim 54, wherein said

(d) virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

57. The method of claim 54, wherein said antibody is a human, humanized, part-human, chimeric, bispecific, recombinant or engineered antibody, or an antigen-binding fragment thereof.

58. The method of claim 54, wherein said pharmaceutical composition is administered to said mammal intravenously.

59. The method of claim 54, wherein said pharmaceutical composition is administered to said mammal as an aerosol.

60. The method of claim 54, wherein said mammal has, or is at risk for developing, hepatitis, influenza, AIDS, viral pneumonia or respiratory disease, smallpox, CMV mononucleosis or Lassa fever.

61. The method of claim 54, wherein at least a second, distinct anti-viral agent is administered to said mammal.

62. The method of claim 61, wherein said at least a second, distinct anti-viral agent is a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

63. The method of claim 54, wherein said mammal is a human patient.

64. The method of claim 54, wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

65. The method of claim 54, wherein said antibody, or antigen-binding fragment thereof, is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

66. A method of inhibiting virus replication or spread, comprising contacting a population of mammalian cells with an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, in an amount effective to inhibit virus replication or spread in said population of cells; wherein said antibody, or antigen-binding fragment thereof, binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid.

67. The method of claim 66, wherein said

(d) virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

68. The method of claim 67, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

69. The method of claim 67, wherein said population of cells is located within a mammal and said immunoconjugate is administered to said mammal.

70. The method of claim 66, wherein said population of cells is located within a human patient and said immunoconjugate is administered to said human patient.

71. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection; wherein said antibody, or antigen-binding fragment thereof, binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid.

72. The method of claim 68, wherein said viral infection is an infection with a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

73. The method of claim 68, wherein said viral infection is an infection with a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

74. The method of claim 71, wherein said mammal is a human patient.

75. A composition comprising a first anti-viral agent, wherein said first anti-viral agent is an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; and at least a second, distinct anti-viral agent; wherein said second, distinct anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor or a non-nucleoside reverse transcriptase inhibitor.

76. The composition of claim 75, wherein said composition further comprises a protease inhibitor.

77. The composition of claim 75, wherein said composition is a pharmaceutical composition.

78. A pharmaceutical composition comprising a first anti-viral agent, wherein said first anti-viral agent is an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; and at least a second, distinct anti-viral agent; wherein said second, distinct anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor or a non-nucleoside reverse transcriptase inhibitor.

79. The pharmaceutical composition of claim 78, wherein said composition further comprises a protease inhibitor.

80. A kit comprising, in at least a first container, at least a first anti-viral agent, wherein said first anti-viral agent is an antibody, or antigen-binding fragment thereof that binds to an aminophospholipid in the absence of serum and serum proteins; and at least a second, distinct anti-viral agent; wherein said second, distinct anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor or a non-nucleoside reverse transcriptase inhibitor.

81. The kit of claim 80, wherein said antibody is a human, humanized, part-human, chimeric, bispecific, recombinant or engineered antibody, or an antigen-binding fragment thereof.

82. The kit of claim 80, wherein said kit further comprises a protease inhibitor.

83. The kit of claim 80, wherein at least one of said first anti-viral agent or said second, distinct anti-viral agent is formulated for intravenous administration.

84. The kit of claim 80, wherein at least one of said first anti-viral agent or said second, distinct anti-viral agent is formulated for administration as an aerosol.

85. The kit of claim 80, wherein said first anti-viral agent and said second, distinct anti-viral agent are comprised within a single container.

86. The kit of claim 80, wherein said first anti-viral agent and said second, distinct anti-viral agent are comprised within distinct containers.

87. A kit comprising, in at least a first container, at least a first anti-viral agent, wherein said first anti-viral agent is an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; and at least a second, distinct anti-viral agent; wherein said second, distinct anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor; and wherein at least one of said first anti-viral agent or said second, distinct anti-viral agent is formulated for administration as an aerosol.

88. A composition comprising a first anti-viral agent and at least a second, distinct anti-viral agent; wherein said first anti-viral agent is an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; and wherein said at least a second, distinct anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor or a non-nucleoside reverse transcriptase inhibitor.

89. A pharmaceutical composition comprising a first anti-viral agent and at least a second, distinct anti-viral agent; wherein said first anti-viral agent is an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; and wherein said at least a second, distinct anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor or a non-nucleoside reverse transcriptase inhibitor.

90. A kit comprising, in at least a first container, at least a first anti-viral agent and at least a second, distinct anti-viral agent; wherein said first anti-viral agent is an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; and wherein said at least a second, distinct anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor or a non-nucleoside reverse transcriptase inhibitor.

91. An immunoconjugate comprising at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, tat binds to an aminophospholipid in the absence of serum and serum proteins; wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

92. The immunoconjugate of claim 91, wherein said antibody is a human, humanized, part-human, chimeric, bispecific, recombinant or engineered antibody, or an antigen-binding fragment thereof.

93. The immunoconjugate of claim 91, wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor or a non-nucleoside reverse transcriptase inhibitor.

94. The immunoconjugate of claim 91, wherein said antibody, or antigen-binding fragment thereof, is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

95. The immunoconjugate of claim 91, wherein said immunoconjugate is comprised in a pharmaceutically acceptable composition.

96. A composition comprising a biologically effective amount of an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

97. A pharmaceutical composition comprising a pharmaceutically acceptable cater and a therapeutically effective amount of an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

98. The pharmaceutical composition of claim 97, wherein said antibody is a human, humanized, part-human, chimeric, bispecific, recombinant or engineered antibody, or an antigen-binding fragment thereof.

99. The pharmaceutical composition of claim 97, wherein said antibody, or antigen-binding fragment thereof, is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

100. The pharmaceutical composition of claim 97, wherein said pharmaceutical composition is formulated for intravenous administration.

101. The pharmaceutical composition of claim 97, wherein said pharmaceutical composition is formulated for administration as an aerosol.

102. A composition comprising a first anti-viral agent, wherein said first anti-viral agent is an anti-viral immunoconjugate comprising one or more anti-viral drugs operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; and at least a second anti-viral agent; wherein said one or more anti-viral drugs or said second anti-viral agent are selected from the group consisting of a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor and a protease inhibitor.

103. The composition of claim 102, wherein said composition is a pharmaceutical composition.

104. A pharmaceutical composition comprising a therapeutically effective amount of a first anti-viral agent, wherein said first anti-viral agent is an anti-viral immunoconjugate comprising one or more anti-viral drugs operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; in combination with a therapeutically effective amount of at least a second anti-viral agent; wherein said one or more anti-viral drugs or said second anti-viral agent are selected from the group consisting of a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor and a protease inhibitor.

105. A kit comprising, in at least a first container, at least a first anti-viral agent, wherein said first anti-viral agent is an anti-viral immunoconjugate comprising one or more anti-viral drugs operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid in the absence of serum and serum proteins; and at least a second anti-viral agent; wherein said one or more anti-viral drugs or said second anti-viral agent are selected from the group consisting of a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor and a protease inhibitor.

106. The kit of claim 105, wherein said antibody is a human, humanized, part-human, chimeric, bispecific, recombinant or engineered antibody, or an antigen-binding fragment thereof.

107. The kit of claim 105, wherein said antibody, or antigen-binding fragment thereof, is operatively attached to said one or more anti-viral drugs via a biologically releasable bond or selectively cleavable linker.

108. The kit of claim 105, wherein said one or more anti-viral drugs or said second anti-viral agent are selected from the group consisting of a nucleoside, a nucleoside reverse transcriptase inhibitor and a non-nucleoside reverse transcriptase inhibitor.

109. The kit of claim 105, wherein at least one of said first anti-viral agent or said second, distinct anti-viral agent is formulated for intravenous administration.

110. The kit of claim 105, wherein at least one of said first anti-viral agent or said second, distinct anti-viral agent is formulated for administration as an aerosol.

111. The kit of claim 105, wherein said first anti-viral agent and said second, distinct anti-viral agent are comprised within a single container.

112. The kit of claim 105, wherein said first anti-viral agent and said second, distinct anti-viral agent are comprised within distinct containers.

113. An immunoconjugate comprising at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

114. A composition comprising a biologically effective amount of an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

115. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of an immunoconjugate that comprises at least a first anti-viral agent operatively attached to an antibody, or antigen-binding fragment thereof that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; wherein said at least a first anti-viral agent is a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

116. A composition comprising a first anti-viral agent, wherein said first anti-viral agent is an anti-viral immunoconjugate comprising one or more anti-viral drugs operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; and at least a second anti-viral agent; wherein said one or more anti-viral drugs or said second anti-viral agent are selected from the group consisting of a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor and a protease inhibitor.

117. A pharmaceutical composition comprising a therapeutically effective amount of a first anti-viral agent, wherein said first anti-viral agent is an anti-viral immunoconjugate comprising one or more anti-viral drugs operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; in combination with a therapeutically effective amount of at least a second anti-viral agent; wherein said one or more anti-viral drugs or said second anti-viral agent are selected from the group consisting of a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor and a protease inhibitor.

118. A kit comprising, in at least a first container, at least a first anti-viral agent, wherein said first anti-viral agent is an anti-viral immunoconjugate comprising one or more anti-viral drugs operatively attached to an antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid and does not require serum or a serum protein to bind to said aminophospholipid; and at least a second anti-viral agent; wherein said one or more anti-viral drugs or said second anti-viral agent are selected from the group consisting of a nucleoside, a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor and a protease inhibitor.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,455,833 B2
APPLICATION NO. : 10/642120
DATED : November 25, 2008
INVENTOR(S) : Philip E. Thorpe, Melina Soares and Sophia Ran

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In claim 17 at column 228, line 43, insert a comma after --thereof--.

In claim 22 at column 228, line 61, delete "Lass a" and insert --Lassa-- therefor.

In claim 27 at column 229, line 17, delete "(d)".

In claim 32 at column 229, line 38, delete "(d)".

In claim 38 at column 229, line 63, delete "(d)".

In claim 42 at column 230, line 15, delete "(d)".

In claim 45 at column 230, line 33, delete "(d)".

In claim 48 at column 230, line 50, delete "(d)".

In claim 56 at column 231, line 18, delete "(d)".

In claim 67 at column 231, line 59, delete "(d)".

In claim 71 at column 232, line 8, insert a comma after --thereof.--.

In claim 80 at column 232, line 48, insert a comma after --thereof.--.

In claim 91 at column 233, line 43, delete "tat" and insert --that-- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,455,833 B2
APPLICATION NO. : 10/642120
DATED : November 25, 2008
INVENTOR(S) : Philip E. Thorpe, Melina Soares and Sophia Ran

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In claim 97 at column 234, line 5, delete "cater" and insert --carrier-- therefor.

In claim 115 at column 235, line 43, insert a comma after --thereof.--.

Signed and Sealed this

Thirteenth Day of January, 2009

JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT E

EXHIBIT E

US007422865B2

(12) **United States Patent**
Fischer

(10) **Patent No.: US 7,422,865 B2**
(45) **Date of Patent: Sep. 9, 2008**

(54) **METHOD OF IDENTIFYING PEPTIDES IN A PROTEOMIC SAMPLE**

(75) Inventor: **Steven M. Fischer**, Hayward, CA (US)

(73) Assignee: **Agilent Technologies, Inc.**, Santa Clara, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 485 days.

(21) Appl. No.: **10/341,310**

(22) Filed: **Jan. 13, 2003**

(65) **Prior Publication Data**

US 2004/0137552 A1 Jul. 15, 2004

(51) **Int. Cl.**

C12Q 1/37	(2006.01)
C07K 1/00	(2006.01)
C07K 17/06	(2006.01)
C07K 17/08	(2006.01)

(52) **U.S. Cl.** **435/23**; 530/402; 530/412; 530/815; 530/816

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner—David M Naff

(57) **ABSTRACT**

The present invention provides a new and improved method for reducing the complexity of a proteomic sample, and preferably also for allowing identification of proteins in the sample. In one aspect, the invention provides a highly efficient method for identifying proteins in a proteomic sample by characterizing a single N-terminal peptide per protein. In another aspect, the invention provides a method for identifying proteins in a proteomic sample by characterizing a single C-terminal peptide per protein. In another aspect, the present invention provides a method for quantitative determination of differential protein expression and/or modification in different samples. In another aspect, the invention relates to kits useful for conveniently performing a method in accordance with the invention.

33 Claims, 1 Drawing Sheet

N-terminal peptides may be lyophilized (e.g., on speedvac). Alternatively, the fractions may be concentrated on a reverse-phase chromatography column. The peptide residue is analyzed by LC/MS. The peptides are fragmented and their MS fragmentation patterns are used to screen available databases to determine the amino acid sequence of the terminal peptides. The amino acid sequence information may then be used to screen protein databases to identify the parent proteins from which the terminal peptides may be derived.

3. Comparative Differential Protein Expression in Two Proteomic Samples Using the Inventive N-terminal Peptide Selection Approach—Differential Isotopic Labeling Prior to Protein Enzymatic Cleavage.

In a exemplary embodiment, protein samples are obtained from two cell states (e.g., diseased vs. normal cell or stressed vs. normal cell). Each sample is treated with 100-1000 molar excess of O-methylisourea in H_2O at pH 9, 50° C. for 2 hours, thereby selectively protecting the protein lysine residues and producing two trypsin cleavable protein mixtures (the reaction mixture may be adjusted to pH 9 by addition of ammonium hydroxide). In one sample, protein N-termini are protected with acetic anhydride-d(0) (alternatively BOC-ON-(d0) can be used) under suitable conditions. In the second sample, protein N-termini are protected with BOC-ON-(d9) under suitable conditions. For example, suitable reaction conditions for protection of the protein N-terminal free amino groups in each sample include reacting with an excess acetic anhydride-d(0) or -d(6) (e.g., 10-100 molar excess) in H_2O at pH 9, 50° C. for 2 hours. The resulting samples are combined, and the combined sample is then subjected to trypsin digestion (e.g., reaction with a trypsin solution buffered to pH 7.5 at 37° C. for 15 hours). The resulting peptide mixture is then exposed to a DITC-modified solid support, thereby effecting immobilization of peptides carrying a free and reactive N-terminal on the solid surface. After sufficient washing of the DITC-modified surface with a suitable solvent (e.g., H_2O/AcCN/AcOH v/v 50/50/0.2), the desired differentially isotopically labeled N-terminal peptides are collected in the washes. If desired, The solvent fractions containing the desired N-terminal peptides may be separately lyophilized; e.g., on speedvac (Alternatively, the fractions may be separately concentrated on a reverse-phase chromatography column). An aliquot is analyzed by LC/MS and the differential amounts of proteins in the original samples can be determined by measuring the relative amounts of each differentially isotopically labeled peptide in the mixture. If tandem MS is used, the amino acid sequence of each peptide in the mixture can be determined, and the identity of the corresponding protein in the original samples can be established by database searching.

4. Comparative Differential Protein Expression in Two Proteomic Samples Using the Inventive N-terminal Peptide Selection Approach—Differential Isotopic Labeling After Protein Enzymatic Cleavage.

In a exemplary embodiment, protein samples are obtained from two cell states (e.g., diseased vs. normal cell or stressed vs. normal cell). Each sample is treated with 100-1000 molar excess of O-methylisourea in H_2O at pH 9, 50° C. for 2 hours, thereby selectively protecting the protein lysine residues and producing two trypsin cleavable protein mixtures (the reaction mixture may be adjusted to pH 9 by addition of ammonium hydroxide). For each sample, protection of the protein N-terminal free amino groups is accomplished by reacting with an excess acetic anhydride (e.g., 10-100 molar excess) in H_2O at pH 9, 50° C. for 2 hours. Each sample is then subjected to trypsin digestion by reacting each N-terminally protected protein mixture with a trypsin solution buffered to pH 7.5 at 37° C. for 15 hours, thereby producing two peptide mixtures. Each resulting peptide mixture is then separately exposed to a DITC-modified solid support, thereby effecting immobilization of peptides carrying a free and reactive N-terminal on the solid surface. After sufficient washing of each DITC-modified surface with a suitable solvent (e.g., H_2O/AcCN/AcOH v/v 50/50/0.2), the desired N-terminally protected peptides are collected in the washes, separately for each sample. The solvent fractions containing the desired N-terminal peptides may be separately lyophilized (e.g., on speedvac). Alternatively, the fractions may be separately concentrated on a reverse-phase chromatography column. In one sample, the peptide free C-termini are activated with a suitable carbodiimide reagent and subsequently reacted with methylamine-(d0). In the second sample, the peptide free C-termini are activated with a suitable carbodiimide reagent and subsequently reacted with methylamine-(d3). The samples are then combined, and the resulting mixture of differentially isotopically labeled peptides is analyzed by LC/MS. The differential amounts of proteins in the original samples can be determined by measuring the relative amounts of each differentially isotopically labeled peptide in the mixture. If tandem MS is used, the amino acid sequence of each peptide in the mixture can be determined, and the identity of the corresponding protein in the original samples can be established by database searching.

What is claimed is:

1. A method for identifying proteins in a proteomic sample comprising the steps of:

a. providing a mixture of proteins;

b. protecting terminal amino or carboxy groups of the proteins with a protecting agent;

c. selectively protecting side chain amino or carboxyl groups of the proteins with a second protecting agent;

d. cleaving the protected proteins with a cleaving agent, thereby producing a peptide mixture of:

(i) terminally protected peptides; and

(ii) non-terminally protected peptides comprising free terminal amino and carboxyl groups corresponding to the cleavage sites;

e. separating the terminally protected peptides from the peptide mixture, thereby reducing the sample complexity to one peptide per sample protein, wherein said separating step e comprises:

(i) selectively covalently bonding the free terminal amino group of the non-terminally protected peptides to a solid support, washing said solid support with a solvent and collecting those solvent fractions containing the terminally protected peptides; or

(ii) wherein the protecting agent of the terminally protected peptides contains a reactive group that can form a covalent bond with a solid support, and selectively covalently bonding the reactive group of the protecting agent to a solid support, washing said solid support with a solvent and releasing the terminally protected peptides from said solid support; and

f. detecting the terminally protected peptides.

2. The method of claim **1** wherein the step of detecting uses mass spectrometric techniques.

3. The method of claim **2** wherein the mass spectrometric technique is coupled to a separation technique.

4. The method of claim **3** wherein the separation technique is high pressure liquid chromatography (HPLC), gel electrophoresis or capillary electrophoresis (CE).

5. The method of claim **3** or **4** wherein the mass spectrometric technique is tandem mass spectrometry (MS).

6. The method of claim 5 wherein said method comprises employing tandem mass spectrometry to produce terminally protected peptide MS fragmentation patterns and said method further comprises employing terminally protected peptide MS fragmentation patterns to screen available databases to determine the amino acid sequence of the terminal peptides.

7. The method of claim 6 wherein the amino acid sequence of the terminal peptides is used to screen protein databases to identify the parent proteins from which the terminal peptides may be derived.

8. The method of claim 1 wherein steps b and c are simultaneously performed.

9. The method of claim 1 wherein, in the step of cleaving, the cleaving agent is an enzyme.

10. The method of claim 9 wherein the enzyme is trypsin, chymotrypsin, pepsin, papain, proline endopeptidase, staph protease, elastase, protease K, protease AspN, protease Lys-C, protease Arg-C or protease Glu-C.

11. The method of claim 9 wherein the enzyme is trypsin.

12. The method of claim 1 wherein in the step of cleaving, the cleaving agent is a chemical cleaving agent.

13. The method of claim 12 wherein the chemical cleaving agent is cyanogen bromide (CNBr), 2-nitro-5-thiocyanobenzoic acid, N-bromosuccinamide and other reactive halogen compounds, hydroxylamine, 1-2M formic or acetic acid, periodate oxidation, 2-(2-nitrophenylsulfenyl) -3-methyl-3-bromoindolenine or oiodosobenzoic acid.

14. The method of claim 1 wherein, in the step of protecting, the protecting agent comprises a radiolabel, a fluorescent label, a colorimetric label or an isotopic label.

15. The method of claim 1 wherein the protecting agent is an amine protecting agent.

16. The method of claim 15 wherein the amine protecting agent is acetic anhydride, which forms an amide moiety upon reacting with a terminal amino group.

17. The method of claim 15 wherein the protecting agent is Boc anhydride or a Fmoc reagent, which forms a Boc or Fmoc carbamate moiety upon reacting with a terminal amino group.

18. The method of claim 15 wherein lysine side chain residues in the proteins are protected with the second protecting agent.

19. The method of claim 18 wherein the second protecting agent is O-methyl-isourea or O-methyl imidazole.

20. The method of claim 1 wherein the step of separating comprises steps of:

a. selectively covalently bonding the free terminal amino group of the non-terminally protected peptides to a solid support;

b. washing the solid support with a solvent; and

c. collecting those solvent fractions containing the terminally protected peptides.

21. The method of claim 20 wherein the solid support is not an affinity label-modified support.

22. The method of claim 20 further comprising a step of releasing the covalently bonded non-terminally protected peptides from the solid support with a releasing agent.

23. The method of claim 20 wherein the non-terminally protected peptides are non-N-terminally protected peptides, and the solid support comprises reactive groups that can form a covalent bond with amine groups.

24. The method of claim 23 wherein the solid support is a diisothiocyante (DITC)-modified support.

25. The method of claim 22 wherein the solid support is a diisothiocyante (DITC)-modified support and the releasing agent is an anhydrous strong acid.

26. The method of claim 25 wherein the anhydrous strong acid is trifluoroacetic acid (TFA), hydrochloric acid (HCl), or heptafluorobutanoic acid (HFBA).

27. The method of claim 20 wherein the non-terminally protected peptides are non-protected C-terminal peptides, and the solid support comprises reactive groups that can form a covalent bond with carboxyl groups.

28. The method of claim 27 wherein the solid support reactive groups are amino groups.

29. The method of claim 27 wherein the step of selectively covalently bonding involves activation of the free carboxyl groups with a carbodiimide reagent.

30. The method of claim 20 wherein, in the step of selectively covalently bonding, the free terminal group is covalently linked to the solid support indirectly through a linker.

31. The method of claim 30 wherein the linker is a photochemically, chemically or enzymatically cleavable linker.

32. The method of claim 1 wherein the step of separating comprises steps of:

a. selectively covalently bonding the reactive group of the protecting agent of the terminally protected peptides to a solid support;

b. washing the solid support with a solvent; and

c. releasing the terminally protected peptides from the solid support.

33. The method of claim 32 wherein, in the step of selectively covalently bonding, the reactive group of the protecting agent is covalently linked to the solid support indirectly through a linker.

* * * * *

EXHIBIT F

EXHIBIT F

US007384909B2

(12) **United States Patent**
Thorpe et al.

(10) **Patent No.: US 7,384,909 B2**
(45) **Date of Patent: Jun. 10, 2008**

(54) **ANTI-VIRAL TREATMENT METHODS USING PHOSPHATIDYLETHANOLAMINE-BINDING PEPTIDES LINKED TO ANTI-VIRAL AGENTS**

(75) Inventors: **Philip E. Thorpe**, Dallas, TX (US); **M. Melina Soares**, Richardson, TX (US); **Jin He**, Dallas, TX (US)

(73) Assignee: **Board of Regents, The University of Texas System**, Austin, TX (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 329 days.

(21) Appl. No.: **10/642,100**

(22) Filed: **Aug. 15, 2003**

(65) **Prior Publication Data**

US 2005/0025761 A1 Feb. 3, 2005

Related U.S. Application Data

(63) Continuation-in-part of application No. 10/621,269, filed on Jul. 15, 2003.

(60) Provisional application No. 60/396,263, filed on Jul. 15, 2002.

(51) **Int. Cl.**
A61K 38/00 (2006.01)
A61K 31/00 (2006.01)

(52) **U.S. Cl.** **514/2**

(58) **Field of Classification Search** 530/350; 514/2

See application file for complete search history.

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Primary Examiner—Larry Helms
Assistant Examiner—Laura B Goddard
(74) *Attorney, Agent, or Firm*—Shelley P. M. Fussey

(57) **ABSTRACT**

Disclosed are surprising discoveries concerning the role of anionic phospholipids and aminophospholipids in tumor vasculature and in viral entry and spread, and compositions and methods for utilizing these findings in the treatment of cancer and viral infections. Also disclosed are advantageous antibody, immunoconjugate and duramycin-based compositions and combinations that bind and inhibit anionic phospholipids and aminophospholipids, for use in the safe and effective treatment of cancer, viral infections and related diseases.

64 Claims, 53 Drawing Sheets

-continued

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<400> SEQUENCE: 9

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Asn Xaa Lys

What is claimed is:

1. A method for inhibiting virus replication, spread or ongoing infection of additional cells or tissues, comprising contacting a mammalian cell with a phosphatidylethanolamine (PE) binding peptide conjugate, which comprises a PE-binding peptide operatively attached to at least a first anti-viral agent, in an amount effective to inhibit virus replication in said cell or inhibit spread or ongoing infection of additional cells or tissues from said cell; wherein said PE-binding peptide is a lantibiotic peptide or a kininogen.

2. The method of claim 1, wherein said PE-binding peptide is a lantibiotic peptide.

3. The method of claim 2, wherein said PE-binding peptide is a cinnamycin peptide.

4. The method of claim 2, wherein said PE-binding peptide is a duramycin peptide.

5. The method of claim 1, wherein said at least a first anti-viral agent is a nucleoside.

6. The method of claim 1, wherein said at least a first anti-viral agent is a reverse transcriptase inhibitor.

7. The method of claim 1, wherein said at least a first anti-viral agent is a protease inhibitor.

8. The method of claim 1, wherein said at least a first anti-viral agent is acyclovir, penciclovir (famciclovir), gancyclovir (ganciclovir), deoxyguanosine, foscarnet, idoxuridine, trifluorothymidine, vidarabine, sorivudine, zidovudine, didanosine, zalcitabine, lamivudine, stavudine, abacavir, multinucleoside resistance A, multinucleoside resistance B, nevirapine, delavirdine, efavirenz, adefovir dipivoxil, indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, deoxycytosine triphosphate, lamivudine triphosphate, emticitabine triphosphate, adefovir diphosphate, penciclovir triphosphate, lobucavir triphosphate, amantadine, rimantadine, zanamivir or oseltamivir.

9. The method of claim 1, wherein said at least a first anti-viral agent is a AZT or cidofovir.

10. The method of claim 1, wherein said PE-binding peptide is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

11. The method of claim 1, wherein said cell is a human cell.

12. The method of claim 1, wherein said cell is infected with a hepatitis, influenza, HIV, herpes, paramyxovirus or arenavirus.

13. The method of claim 1, wherein said method inhibits viral replication.

14. The method of claim 1, wherein said method inhibits viral spread.

15. The method of claim 1, wherein said cell is located within a mammal and said PE-binding peptide conjugate is administered to said mammal.

16. The method of claim 15, wherein said mammal is a human patient.

17. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising a PE-binding peptide conjugate, which comprises a PE-binding peptide operatively attached

to at least a first anti-viral agent, in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection; wherein said PE-binding peptide is a lantibiotic peptide or a kininogen.

18. The method of claim **17**, wherein said pharmaceutical composition is administered to said mammal intravenously.

19. The method of claim **17**, wherein said pharmaceutical composition is administered to said mammal as an aerosol.

20. The method of claim **17**, wherein said mammal has, or is at risk for developing, hepatitis, influenza, AIDS, viral pneumonia or respiratory disease or Lassa fever.

21. The method of claim **17**, wherein at least a second, distinct anti-viral agent is administered to said mammal.

22. The method of claim **21**, wherein said at least a second, distinct anti-viral agent is a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

23. The method of claim **17**, wherein said mammal is a human patient.

24. The method of claim **1**, wherein said PE-binding peptide is a kininogen.

25. The method of claim **17**, wherein said PE-binding peptide is a kininogen.

26. The method of claim **17**, wherein said PE-binding peptide is a lantibiotic peptide.

27. The method of claim **26**, wherein said PE-binding peptide is a cinnamycin peptide.

28. The method of claim **26**, wherein said PE-binding peptide is a duramycin peptide.

29. The method of claim **17**, wherein said at least a first anti-viral agent is a nucleoside, a reverse transcriptase inhibitor or a protease inhibitor.

30. The method of claim **17**, wherein said at least a first anti-viral agent is AZT, cidofovir, acyclovir, penciclovir (famciclovir), gancyclovir (ganciclovir), deoxyguanosine, foscarnet, idoxuridine, trifluorothymidine, vidarabine, sorivudine, zidovudine, didanosine, zalcitabine, lamivudine, stavudine, abacavir, multinucleoside resistance A, multinucleoside resistance B, nevirapine, delavirdine, efavirenz, adefovir dipivoxil, indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, deoxycytosine triphosphate, lamivudine triphosphate, emticitabine triphosphate, adefovir diphosphate, penciclovir triphosphate, lobucavir triphosphate, amantadine, rimantadine, zanamivir or oseltamivir.

31. The method of claim **17**, wherein said PE-binding peptide is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

32. A method for inhibiting virus replication, spread or ongoing infection of additional cells or tissues, comprising contacting a mammalian cell with a phosphatidylethanolamine (PE) binding peptide conjugate in an amount effective to inhibit virus replication in said cell or inhibit spread or ongoing infection of additional cells or tissues from said cell; wherein said PE-binding peptide conjugate comprises at least a first anti-viral agent operatively attached to a lantibiotic peptide that binds to PE or to a kininogen that binds to PE; and wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

33. The method of claim **32**, wherein said lantibiotic peptide that binds to PE is a cinnamycin peptide.

34. The method of claim **32**, wherein said lantibiotic peptide that binds to PE is a duramycin peptide.

35. The method of claim **32**, wherein said at least a first anti-viral agent is a nucleoside, a reverse transcriptase inhibitor or a protease inhibitor.

36. The method of claim **32**, wherein said at least a first anti-viral agent is AZT, cidofovir, acyclovir, penciclovir (famciclovir), gancyclovir (ganciclovir), deoxyguanosine, foscarnet, idoxuridine, trifluorothymidine, vidarabine, sorivudine, zidovudine, didanosine, zalcitabine, lamivudine, stavudine, abacavir, multinucleoside resistance A, multinucleoside resistance B, nevirapine, delavirdine, efavirenz, adefovir dipivoxil, indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, deoxycytosine triphosphate, lamivudine triphosphate, emticitabine triphosphate, adefovir diphosphate, penciclovir triphosphate, lobucavir triphosphate, amantadine, rimantadine, zanamivir or oseltamivir.

37. The method of claim **32**, wherein said lantibiotic peptide that binds to PE is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

38. The method of claim **32**, wherein said cell is a human cell.

39. The method of claim **32**, wherein said cell is infected with a hepatitis, influenza, HIV, herpes, paramyxovirus or arenavirus.

40. The method of claim **32**, wherein said method inhibits viral replication.

41. The method of claim **32**, wherein said method inhibits viral spread.

42. The method of claim **32**, wherein said cell is located within a mammal and said PE-binding peptide conjugate is administered to said mammal.

43. The method of claim **42**, wherein said mammal is a human patient.

44. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising a PE-binding peptide conjugate in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection; wherein said PE-binding peptide conjugate comprises at least a first anti-viral agent operatively attached to a lantibiotic peptide that binds to PE or to a kininogen that binds to PE; and wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

45. The method of claim **44**, wherein said lantibiotic peptide that binds to PE is a cinnamycin peptide.

46. The method of claim **44**, wherein said lantibiotic peptide that binds to PE is a duramycin peptide.

47. The method of claim **44**, wherein said at least a first anti-viral agent is a nucleoside, a reverse transcriptase inhibitor or a protease inhibitor.

48. The method of claim **44**, wherein said at least a first anti-viral agent is AZT, cidofovir, acyclovir, penciclovir (famciclovir), gancyclovir (ganciclovir), deoxyguanosine, foscarnet, idoxuridine, trifluorothymidine, vidarabine, sorivudine, zidovudine, didanosine, zalcitabine, lamivudine, stavudine, abacavir, multinucleoside resistance A, multinucleoside resistance B, nevirapine, delavirdine, efavirenz, adefovir dipivoxil, indinavir, ritonavir, saquinavir, nelfinavir, amprenavir, deoxycytosine triphosphate, lamivudine triphosphate, emticitabine triphosphate, adefovir diphosphate, penciclovir triphosphate, lobucavir triphosphate, amantadine, rimantadine, zanamivir or oseltamivir.

49. The method of claim **44**, wherein said lantibiotic peptide that binds to PE is operatively attached to said at least a first anti-viral agent via a biologically releasable bond or selectively cleavable linker.

50. The method of claim 44, wherein said pharmaceutical composition is administered to said mammal intravenously.

51. The method of claim 44, wherein said pharmaceutical composition is administered to said mammal as an aerosol.

52. The method of claim 44, wherein said mammal has, or is at risk for developing, hepatitis, influenza, AIDS, viral pneumonia or respiratory disease or Lassa fever.

53. The method of claim 44, wherein at least a second, distinct anti-viral agent is administered to said mammal.

54. The method of claim 53, wherein said at least a second, distinct anti-viral agent is a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor.

55. The method of claim 44, wherein said mammal is a human patient.

56. The method of claim 32, wherein said PE-binding peptide conjugate comprises a kininogen that binds to PE.

57. The method of claim 32, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

58. The method of claim 44, wherein said PE-binding peptide conjugate comprises a kininogen that binds to PE.

59. The method of claim 44, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

60. A method for inhibiting virus replication, spread or ongoing infection of additional cells or tissues, comprising contacting a mammalian cell with a phosphatidylethanolamine (PE) binding peptide conjugate in an amount effective to inhibit virus replication in said cell or inhibit spread or ongoing infection of additional cells or tissues from said cell; wherein said PE-binding peptide conjugate comprises at least a first anti-viral agent operatively attached to a lantibiotic peptide that binds to PE; and wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramyxoviridae, Poxviridae, Retroviridae or Rhabdoviridae family.

61. The method of claim 60, wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

62. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising a PE-binding peptide conjugate in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection; wherein said PE-binding peptide conjugate comprises at least a first anti-viral agent operatively attached to a lantibiotic peptide that binds to PE; and wherein said virus is a virus from the Arenaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae or Rhabdoviridae family.

63. A method for inhibiting virus replication, spread or ongoing infection of additional cells or tissues, comprising contacting a mammalian cell with a phosphatidylethanolamine (PE) binding peptide conjugate in an amount effective to inhibit virus replication in said cell or inhibit spread or ongoing infection of additional cells or tissues from said cell; wherein said PE-binding peptide conjugate comprises at least a first anti-viral agent operatively attached to a lantibiotic peptide that binds to PE; and wherein said virus is a virus from the Herpesviridae family.

64. A method for treating a mammal with a viral infection, comprising administering to said mammal a pharmaceutical composition comprising a PE-binding peptide conjugate in an amount effective to inhibit viral replication or spread in said mammal, thereby treating said viral infection; wherein said PE-binding peptide conjugate comprises at least a first anti-viral agent operatively attached to a lantibiotic peptide that binds to PE; and wherein said virus is a virus from the Herpesviridae family.

* * * * *

EXHIBIT G

EXHIBIT G

US007279564B2

(12) **United States Patent**
De Nobel et al.

(10) **Patent No.: US 7,279,564 B2**
(45) **Date of Patent: Oct. 9, 2007**

(54) **EXPRESSION IN FILAMENTOUS FUNGI OF PROTEASE INHIBITORS AND VARIANTS THEREOF**

(75) Inventors: **Hans De Nobel**, Almere (NL); **David A. Estell**, San Mateo, CA (US); **Wei Liu**, Palo Alto, CA (US); **Scott D. Power**, San Bruno, CA (US); **Brian Schmidt**, Half Moon Bay, CA (US); **Huaming Wang**, Fremont, CA (US)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 59 days.

(21) Appl. No.: **10/971,596**

(22) Filed: **Oct. 22, 2004**

(65) **Prior Publication Data**

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(51) **Int. Cl.**

C07H 21/02	(2006.01)
C12N 15/00	(2006.01)
C12N 1/15	(2006.01)

(52) **U.S. Cl.** **536/23.1**; 435/320.1; 435/254.11; 435/71.1

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner—Cecilia J. Tsang
Assistant Examiner—Christina M. Bradley

(57) **ABSTRACT**

Described herein are protease inhibitors, variants thereof and methods for their production.

23 Claims, 12 Drawing Sheets

-continued

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Asn Val Ile Ser Lys Arg
1 5

The invention claim is:

1. A method for producing a protease inhibitor in a filamentous fungal cell comprising,

a) introducing a DNA construct into a filamentous fungal cell, wherein said DNA construct comprises a promoter showing transcription activity in the filamentous fungal cell and which is operably linked to a heterologous DNA sequence encoding a Bowman-Birk Inhibitor (BBI) comprising SEQ ID NO: 7 wherein residues 15-21 and/or 42-47 have been replaced with a variant sequence or consisting of SEQ ID NO: 7

b) culturing the filamentous fungal cell under suitable culture conditions to allow expression of the heterologous DNA sequence, and

c) producing the protease inhibitor.

2. The method according to claim 1 further comprising recovering the protease inhibitor.

3. The method according to claim 1, wherein the filamentous fungal cell is selected from an *Aspergillus* strain, a *Penicillium* strain, a *Fusarium* strain or a *Trichoderma* strain.

4. The method according to claim 3, wherein the *Trichoderma* strain is *T. reesei.*

5. The method according to claim 3, wherein the *Aspergillus* strain is *A. niger, A. nidulans, A. awamori* or *A. oryzea.*

6. The method according to claim 1 wherein the protease inhibitor is selected from the group consisting of SEQ ID NOs. 7 and 15-29.

7. The method according to claim 1, wherein the DNA sequence encoding the protease inhibitor includes codons that have been optimized for expression in the filamentous fungal cell.

8. The method according to claim 1 further comprising introducing a second nucleic acid sequence encoding a chaperone into the filamentous fungal cell.

9. The method according to claim 8, wherein the chaperone is pdiA or prpA.

10. The method according to claim 1, wherein the protease inhibitor is expressed as a fusion protein.

11. The method according to claim 10, wherein the fusion protein includes a glucoamylase signal sequence, a glucoamylase catalytic domain, a cleavage site, and the protease inhibitor.

12. The method according to claim 10, wherein the fusion protein is processed by a protease to liberate the protease inhibitor.

13. An isolated polynucleotide encoding a protease inhibitor selected from the group consisting of polypeptide sequences set forth in SEQ ID NOs: 15-29.

14. An altered wild-type polynucleotide comprising codons which have been optimized for expression in a filamentous fungal cell encoding a Bowman Birk Inhibitor comprising SEQ ID NO: 7 wherein residues 15-21 and/or 42-47 have been replaced with a variant sequence or consisting of SEQ ID NO: 7.

15. An expression vector comprising

a polynucleotide sequence encoding a Bowman Birk Inhibitor comprising SEQ ID NO: 7 wherein residues 15-21 and/or 42-47 have been replaced with a variant sequence or consisting of SEQ ID NO: 7.

16. The expression vector of claim 15, further comprising from the 5' terminus to the 3' terminus,

a first nucleic acid sequence encoding a signal peptide functional as a secretory sequence in a filamentous fungus,

a second nucleic acid sequence encoding a secreted polypeptide or functional portion thereof,

a third nucleic acid sequence encoding a cleavable linker, and

the DNA sequence which encodes the protease inhibitor.

17. A host cell transformed with the vector of claim 15.

18. The host cell of claim 17, wherein said host cell is a *Triclioderma* cell.

19. The host cell of claim 17, wherein said host cell is an *Aspergillus* cell.

20. A method for enhancing the expression of a protease inhibitor in a filamentous fungal cell comprising

(a) transforming a filamentous fungal cell with a DNA construct which comprises a promoter showing transcription activity in the filamentous fungal cell and which is operably linked to a fusion DNA sequence comprising a heterologous DNA sequence encoding a Bowman-Birk Inhibitor (BBI) comprising SEQ ID NO: 7 wherein residues 15-21 and or 42-47 have been replaced with a variant sequence or consisting of SEQ ID NO: 7,

(b) transforming the filamentous fungal cell with a polynucleotide sequence containing a chaperone gene, and

(c) culturing the filamentous fungal cell under suitable culture conditions to allow expression and secretion of the heterologous DNA sequence encoding the protease inhibitor

wherein expression of the protease inhibitor is enhanced compared to a corresponding filamentous fungal cell transformed only in accordance with step a).

21. The method according to claim 20, wherein the transformation step a) and the transformation step b) is a co-transformation.

22. The method according to claim 20, wherein the transformation step a) and transformation step b) is a sequential transformation.

23. The method according to claim 20, wherein the filamentous fungi cell is an *Aspergillus* cell or a *Trichoderma* cell.

* * * * *

EXHIBIT H

EXHIBIT H

US005679543A

United States Patent [19]

Lawlis

[11] Patent Number: 5,679,543

[45] Date of Patent: Oct. 21, 1997

[54] **DNA SEQUENCES, VECTORS AND FUSION POLYPEPTIDES TO INCREASE SECRETION OF DESIRED POLYPEPTIDES FROM FILAMENTOUS FUNGI**

[75] Inventor: **Virgil Bryan Lawlis**, San Mateo, Calif.

[73] Assignee: **Genencor International, Inc.**, Rochester, N.Y.

[21] Appl. No.: **318,491**

[22] Filed: **Oct. 5, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 207,805, Mar. 7, 1994, abandoned, which is a continuation of Ser. No. 794,603, Nov. 15, 1991, abandoned, which is a continuation of Ser. No. 369,698, Jun. 16, 1989, abandoned, which is a continuation-in-part of Ser. No. 163,219, Feb. 26, 1988, abandoned, which is a continuation of Ser. No. 882,224, Jul. 7, 1986, abandoned, which is a continuation-in-part of Ser. No. 771,374, Aug. 29, 1985, abandoned.

[51] **Int. Cl.**[6] **C12P 21/06**; C12N 15/63; C07K 14/00; C07H 21/04

[52] **U.S. Cl.** **435/69.1**; 435/69.8; 435/71.1; 435/172.3; 435/205; 435/254.11; 435/320.1; 530/350; 536/23.1; 536/23.4; 536/24.1

[58] **Field of Search** 435/69.1, 69.8, 435/71.1, 172.3, 205, 254.1, 320.1; 530/350; 536/23.1, 23.4, 24.1

[56] **References Cited**

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Primary Examiner—George C. Elliott
Assistant Examiner—Thanda Wai
Attorney, Agent, or Firm—Flehr Hohbach Test Albritton & Herbert; Richard F. Trecartin; Robin M. Silva

[57] **ABSTRACT**

The invention includes novel fusion DNA sequences encoding fusion polypeptides which when expressed in a filamentous fungus result in increased levels of secretion of the desired polypeptide as compared to the expression and secretion of such polypeptides from filamentous fungi transformed with previously used DNA sequences. The fusion DNA sequences comprise from the 5' terminus four DNA sequences which encode a fusion polypeptide comprising, from the amino to carbonyl-terminus, first, second, third and fourth amino acid sequences. The first DNA sequence encodes a signal peptide functional as a secretory sequence in a first filamentous fungus. The second DNA sequence encodes a secreted polypeptide or portion thereof which is normally secreted from the same filamentous fungus or a second filamentous fungus. The third DNA sequence encodes a cleavable linker polypeptide while the fourth DNA sequence encodes a desired polypeptide. When the fusion DNA sequence is expressed either in the first or second filamentous fungus, increased secretion of the desired polypeptide is obtained as compared to that which is obtained when the desired polypeptide is expressed from DNA sequences encoding a fusion polypeptide which does not contain the second polypeptide normally secreted from either of the filamentous fungi.

47 Claims, 14 Drawing Sheets

in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

All references are expressly incorporated herein by reference.

What is claimed is:

1. A fusion DNA sequence encoding a fusion polypeptide comprising, from the 5' end of said fusion DNA sequence, first, second, third and fourth DNA sequences encoding, from the amino- to carboxy-terminus of said fusion polypeptide, corresponding first, second, third and fourth amino acid sequences, said first DNA sequence encoding a signal peptide functional as a secretory sequence in a first filamentous fungus, said second DNA sequence encoding a mature form of a secreted polypeptide normally secreted from said first or a second filamentous fungus or portion thereof comprising greater than 50% of the amino terminal sequence of said secreted polypeptide, said third DNA sequence encoding a cleavable linker polypeptide and said fourth DNA sequence encoding a desired polypeptide, wherein said first and said second filamentous fungi are selected from the group consisting of Aspergillus, Trichoderma and Neurospora and the expression of said fusion DNA sequence in said first or said second filamentous fungus results in increased secretion of said desired polypeptide as compared to the secretion of said desired polypeptide from said first or said second filamentous fungus when expressed as a second fusion polypeptide encoded by a second fusion DNA sequence comprising only said first, third and fourth DNA sequences.

2. The fusion DNA sequence of claim 1 wherein said first DNA sequence encodes a signal peptide or portion thereof selected from the group consisting of signal peptides from glucoamylase, α-amylase, and aspartyl protease from Aspergillus spp., signal peptides from bovine chymosin and human tissue plasminogen activator and signal peptides from Trichoderma cellobiohydrolase I and II.

3. The fusion DNA sequence of claim 1 wherein said first DNA sequence encodes the signal peptide from *Aspergillus awamori* glucoamylase.

4. The fusion DNA sequence of claim 1 wherein said second DNA sequence encodes a secreted polypeptide selected from the group consisting of glucoamylase, α-amylase, and aspartyl protease from Aspergillus spp. and Trichoderma cellobiohydrolase I and II.

5. The fusion DNA sequence of claim 1 wherein said second DNA sequence encodes glucoamylase from *Aspergillus awamori*.

6. The fusion DNA sequence of claim 1 wherein said third DNA sequence encodes a cleavable linker polypeptide selected from the group consisting of the prosequence from chymosin, the prosequence of subtilisin, and sequences recognized by trypsin factor X_a, collagenase, clostripain, subtilisin and chymosin.

7. The DNA sequence of claim 1 wherein said third DNA sequence encodes the prosequence of chymosin or a portion thereof.

8. The fusion DNA sequence of claim 1 wherein said fourth DNA sequence encodes a desired polypeptide selected from the group consisting of enzymes, proteinaceous hormones and serum proteins.

9. The fusion DNA sequence of claim 1 wherein said fourth DNA sequence encodes bovine chymosin.

10. The fusion DNA sequence of claim 1 wherein said first DNA sequence encodes the signal peptide from *Aspergillus awamori* glucoamylase, said second sequence encodes glucoamylase from *Aspergillus awamori* said third sequence encodes the prosequence of chymosin and said fourth sequence encodes chymosin.

11. An expression vector for transforming a host filamentous fungus selected from the group consisting of Aspergillus, Trichoderma and Neurospora comprising DNA sequences encoding regulatory sequences functionally recognized by said host filamentous fungus including promoter and transcription and translation initiation sequences operably linked to the 5' end of the fusion DNA sequence of claim 1 and transcription stop sequences and polyadenylation sequences operably linked to the 3' end of said fusion DNA sequence.

12. The expression vector of claim 11 wherein said first and said second DNA sequences encoding respectively said signal peptide and said secreted polypeptide are selected from filamentous fungi of the same genus as said host filamentous fungus.

13. The expression vector of claim 12 wherein said genus is selected from the group consisting of Aspergillus, Trichoderma, and Neurospora.

14. The expression vector of claim 12 wherein said genus is Aspergillus.

15. The expression vector of claim 11 wherein said first and said second DNA sequences encoding respectively said signal peptide and said secreted polypeptide are from said host filamentous fungus.

16. A host filamentous fungus selected from the group consisting of Aspergillus, Trichoderma and Neurospora comprising any one of the expression vectors of claims 11 through 15.

17. A fusion polypeptide comprising, from the amino-to carboxy-terminus, first, second, third and fourth amino acid sequences, said first amino acid sequence comprising a signal peptide functional as a secretory sequence in a first filamentous fungus, said second amino acid sequence comprising a mature form of a secreted polypeptide normally secreted from said first or a second filamentous fungus or portion thereof comprising greater than 50% of the amino terminal sequence of said secreted polypeptide, said third amino acid sequence comprising a cleavable linker polypeptide and said fourth amino acid sequence comprising a desired polypeptide, wherein said first and said second filamentous fungi are selected from the group consisting of Aspergillus, Trichoderma and Neurospora and the expression of the fusion DNA sequence encoding said fusion polypeptide in said first or said second filamentous fungus results in increased secretion of said desired polypeptide as compared to the secretion of said desired polypeptide from said first or said second filamentous fungus when expressed from a second fusion DNA sequence encoding a second fusion polypeptide comprising said first, third and fourth amino acid sequences.

18. The fusion polypeptide of claim 17 wherein said first amino acid sequence comprises a signal peptide or portion thereof selected from the group consisting of signal peptides from glucoamylase, α-amylase, and aspartyl protease from Aspergillus spp., signal peptides from bovine chymosin and human tissue plasminogen activator and signal peptides from Trichoderma cellobiohydrolase I and II.

19. The fusion polypeptide of claim 17 wherein said first amino acid sequence is the signal peptide from *Aspergillus awamori* glucoamylase.

20. The fusion polypeptide of claim 17 wherein said second amino acid sequence is selected from the group consisting of glucoamylase, α-amylase, and aspartyl protease from Aspergillus spp. and Trichoderma cellobiohydrolase I and II.

21. The fusion polypeptide of claim 17 wherein said second amino acid sequence is glucoamylase from *Aspergillus awamori*.

22. The fusion polypeptide of claim 17 wherein said cleavable linker polypeptide is selected from the group consisting of the prosequence of subtilisin, and sequences recognized by trypsin Factor Xa, collagenase, clostripain, subtilisin and chymosin.

23. The fusion polypeptide of claim 17 wherein said third amino acid sequence is the prosequence of chymosin.

24. The fusion polypeptide of claim 17 wherein said fourth amino acid sequence is selected from the group consisting of enzymes, proteinaceous hormones and serum proteins.

25. The fusion polypeptide of claim 17 wherein said fourth amino acid sequence is chymosin.

26. The fusion polypeptide of claim 17 wherein said first amino acid sequence is the signal peptide of *A. awamori* glucoamylase, said second amino acid sequence is glucoamylase from *A. awamori* said third amino acid sequence is the prosequence of chymosin and said fourth amino acid sequence is bovine chymosin.

27. A process for producing a desired polypeptide comprising:

transforming a host filamentous fungus selected from the group consisting of Aspergillus, Trichoderma and Neurospora with an expression vector containing the vector of claim 11 under conditions which permit expression of said fusion DNA sequence to cause the secretion of the desired polypeptide encoded by said fusion DNA sequence.

28. The fusion DNA of claim 1 wherein said first and said second filamentous fungi are from the same genus.

29. The fusion DNA of claim 28 wherein said genus is selected from the group consisting of Aspergillus, Trichoderma, and Neurospora.

30. The fusion DNA of claim 28 wherein said genus comprises Aspergillus.

31. The fusion polypeptide of claim 17 wherein said first and said second filamentous fungi are from the same genus.

32. The fusion polypeptide of claim 31 wherein said genus is selected from the group consisting of Aspergillus, Trichoderma, and Neurospora.

33. The fusion polypeptide of claim 31 wherein said genus comprises Aspergillus.

34. The fusion DNA sequence of claim 1 wherein said portion comprises greater than 75% of the amino acid sequence of said secreted polypeptide.

35. The fusion DNA of claim 1 wherein said portion comprises greater than 90% of the amino acid sequence of said secreted polypeptide.

36. The fusion DNA of claims 1, 34 or 35 wherein said portion of said secreted polypeptide is the amino terminal portion.

37. The fusion polypeptide of claim 17 wherein said portion comprises greater than 75% of the amino acid sequence of said secreted polypeptide.

38. The fusion polypeptide of claim 17 wherein said portion comprises greater than 90% of the amino terminal portion of said secreted polypeptide.

39. The fusion polypeptide of claim 17, 37 or 38 wherein said portion of said secreted polypeptide is the amino terminal portion.

40. A fusion DNA sequence encoding a fusion polypeptide comprising, from the 5' end of said fusion DNA sequence, first, second, third and fourth DNA sequences encoding, from the amino- to carboxy-terminus of said fusion polypeptide, corresponding first, second, third and fourth amino acid sequences, wherein

a) said first DNA sequence encodes a signal peptide functional as a secretory sequence in a first filamentous fungus;

b) said second DNA sequence encodes a mature form of a secreted polypeptide normally secreted from said first or a second filamentous fungus or portion thereof comprising greater than 50% of the amino terminal sequence of said secreted polypeptide, and is selected from the group consisting of glucoamylase, α-amylase and aspartyl proteases from *Aspergillus awamori, Aspergillus niger*, and *Aspergillus oryzae*, cellobiohydrolase I, cellobiohydrolase II, endoglucanase I, and endoglucanase III sequences from Trichoderma, and glucoamylase sequences from Neurospora and Humicola;

c) said third DNA sequence encodes a cleavable linker polypeptide; and

d) said fourth DNA sequence encodes a desired polypeptide;

wherein said first and said second filamentous fungi are selected from the group consisting of Aspergillus, Trichoderma and Neurospora and the expression of said fusion DNA sequence in said first or said second filamentous fungus results in increased secretion of said desired polypeptide as compared to the secretion of said desired polypeptide from said first or said second filamentous fungus when expressed as a second fusion polypeptide encoded by a second fusion DNA sequence comprising only said first, third and fourth DNA sequences.

41. A fusion DNA sequence according to claim 40 wherein said first DNA sequence is selected from the group consisting of glucoamylase, α-amylase and aspartyl protease signal sequences from *Aspergillus awamori, Aspergillus niger*, and *Aspergillus oryzae*, cellobiohydrolase I, cellobiohydrolase II, endoglucanase I, and endoglucanase III signal sequences from Trichoderma, glucoamylase signal sequences from Neurospora and Humicola, bovine chymosin signal sequence, human tissue plasminogen activator signal sequence, human interferon signal sequence, and synthetic consensus eukaryotic signal sequences.

42. A fusion DNA sequence according to claim 44 herein said third DNA sequence is selected from the group consisting of the prosequence of bovine chymosin, the prosequence of subtilisin, the prosequence of human immunodeficiency virus protease, and sequences recognized and cleaved by trypsin, Factor Xa, collagenase, clostripin, subtilisin, chymosin, and yeast KEX2 protease.

43. A fusion DNA sequence encoding a fusion polypeptide according to claim 42 wherein said desired polypeptide is selected from the group consisting of bovine chymosin, human tissue plasminogen activator, human growth hormone, human interferon, human interleukin, human serum albumin, Bacillus α-amylase, Pseudomonas lipase, lignin peroxidase and Mn2+-dependent peroxidase from Phanerochaete, Humicola glucoamylase, and Mucor aspartyl proteases.

44. A fusion polypeptide comprising, from the amino-to carboxy-terminus, first, second, third and fourth amino acid sequences, wherein:

a) said first amino acid sequence comprises a signal peptide functional as a secretory sequence in a first filamentous fungus;

b) said second amino acid sequence comprises a mature form of a secreted polypeptide normally secreted from

said first or a second filamentous fungus or portion thereof comprising greater than 50% of the amino terminal sequence of said secreted polypeptide, and is selected from the group consisting of glucoamylase, α-amylase and aspartyl proteases from *Aspergillus awamori, Aspergillus niger*, and *Aspergillus oryzae*, cellobiohydrolase I, cellobiohydrolase II, endoglucanase I, and endoglucanase III sequences from Trichoderma, and glucoamylase sequences from Neurospora and Humicola;

c) said third amino acid sequence comprises a cleavable linker polypeptide; and

d) said fourth amino acid sequence comprises a desired polypeptide;

wherein said first and said second filamentous fungi are selected from the group consisting of Aspergillus, Trichoderma and Neurospora and the expression of the fusion DNA sequence encoding said fusion polypeptide in said first or said second filamentous fungus results in increased secretion of said desired polypeptide as compared to the secretion of said desired polypeptide from said first or said second filamentous fungus when expressed from a second fusion DNA sequence encoding a second fusion polypeptide comprising said first, third and fourth amino acid sequences.

45. A fusion polypeptide according to claim **44** wherein said first amino acid sequence is selected from the group consisting of glucoamylase, α-amylase and aspartyl protease signal sequences from *Aspergillus awamori, Aspergillus niger*, and *Aspergillus oryzae*, cellobiohydrolase I, cellobiohydrolase II, endoglucanase I, and endoglucanase III signal sequences from Trichoderma, glucoamylase signal sequences from Neurospora and Humicola, bovine chymosin signal sequence, human tissue plasminogen activator signal sequence, human interferon signal sequence, and synthetic consensus eukaryotic signal sequences.

46. A fusion polypeptide according to claim **45** wherein said first amino acid sequence is selected from the group consisting of the prosequence of bovine chymosin, the prosequence of subtilisin, the prosequence of human immunodeficiency virus protease, and sequences recognized and cleaved by trypsin, Factor Xa, collagenase, clostripin, subtilisin, chymosin, and yeast KEX2 protease.

47. A fusion polypeptide according to claim **46** wherein said desired polypeptide is selected from the group consisting of bovine chymosin, human tissue plasminogen activator, human growth hormone, human interferon, human interleukin, human serum albumin, Bacillus α-amylase, Pseudomonas lipase, lignin peroxidase and Mn2+-dependent peroxidase from Phanerochaete, Humicola glucoamylase, and Mucor aspartyl proteases.

* * * * *

EXHIBIT I

EXHIBIT I

US005654176A

United States Patent [19]

Smith

[11] Patent Number: 5,654,176

[45] Date of Patent: Aug. 5, 1997

[54] **FUSION PROTEINS CONTAINING GLUTATHIONE-S-TRANSFERASE**

[75] Inventor: **Donald Bruce Smith**, Berwickshire, Scotland

[73] Assignee: **Amrad Corporation Limited**, Victoria, Australia

[21] Appl. No.: **307,337**

[22] Filed: **Sep. 16, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 865,718, Apr. 8, 1992, abandoned, which is a continuation of Ser. No. 331,521, filed as PCT/AU88/00164, May 27, 1988 published as WO88/09372, Dec. 1, 1988, abandoned .

[30] **Foreign Application Priority Data**

May 28, 1987 [AU] Australia PI2195/87

[51] **Int. Cl.**[6] **C12P 21/00**; C12N 5/10; C12N 15/62; C12N 15/63

[52] **U.S. Cl.** **435/69.7**; 435/193; 435/252.3; 435/252.33; 435/320.1; 435/348; 530/350; 536/23.4

[58] **Field of Search** 435/69.7, 172.3, 435/320.1, 252.3, 69.1, 240.2, 252.33; 536/23.4; 530/350, 300

[56] **References Cited**

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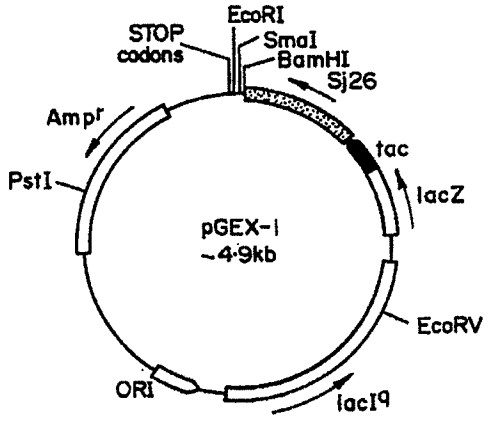
Primary Examiner—Charles L. Patterson, Jr.
Assistant Examiner—Eric Grimes
Attorney, Agent, or Firm—Scully, Scott, Murphy & Presser

[57] **ABSTRACT**

A recombinant DNA molecule comprising a nucleotide sequence which codes for expression of a fusion protein in which a foreign protein or peptide is fused with the enzyme glutathione-S-transferase, is disclosed, as well as expression vectors or host cells containing such a molecule. Optionally, the foreign protein or peptide is fused to the enzyme through a cleavable link. Also disclosed is an expression vector having inserted therein a nucleotide sequence capable of being expressed as the enzyme glutathione-S-transferase followed by at least one restriction endonuclease recognition site for insertion of a nucleotide sequence capable of being expressed as a foreign protein or peptide fused to the glutathione-S-transferase.

25 Claims, 9 Drawing Sheets



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I claim:

1. A recombinant DNA molecule comprising a nucleotide sequence which codes for a fusion protein wherein said fusion protein comprises glutathione-S-transferase and a second protein or peptide fused directly or indirectly with the COOH-terminus of glutathione-S-transferase, and wherein said fusion protein is capable of binding to glutathione.

2. The recombinant DNA molecule according to claim **1**, wherein said glutathione-S-transferase is the 26kDa glutathione-S-transferase of *Schistosoma japonicum.*

3. The recombinant DNA molecule according to claim **1** wherein said second protein or peptide is fused to said glutathione-S-transferase through a cleavable link.

4. The recombinant DNA molecule according to claim **3**, wherein said cleavable link is cleavable by a site specific protease.

5. The recombinant DNA molecule according to claim **4**, wherein said clearable link is cleavable by thrombin, blood coagulation factor X_a or renin.

6. The recombinant DNA molecule of claim **1** wherein said glutathione-S-transferase is a mammalian glutathione-S-transferase.

7. An expression vector comprising the recombinant DNA molecule of any one of claims 1, 2–5 and 6.

8. A host cell comprising the expression vector of claim **7.**

9. The host cell of claim **8** wherein said host cell is a bacterial cell.

10. The host cell according to claim **9** which is *E. coli.*

11. An expression vector comprising, in the 5' to 3' direction, a promoter, a nucleotide sequence encoding glutathione-S-transferase and a nucleotide sequence comprising at least one restriction endonuclease recognition site.

12. The expression vector according to claim **11**, wherein said glutathione-S-transferase is the 26kDa glutathione-S-transferase of *Schistosoma japonicum.*

13. The expression vector according to claim **11**, which comprises a plasmid selected from the group consisting of pGEX-1, pSj10 DBamI and pSj10 DBam7Stop7.

14. An expression vector comprising, in the 5' to 3' direction, a promoter, a nucleotide sequence encoding glutathione-S-transferase, a nucleotide sequence encoding a clearable link and a nucleotide sequence comprising at least one restriction endonuclease recognition site.

15. The expression vector according to claim **14**, wherein said cleavable link is cleavable by a site specific protease.

16. The expression vector according to claim **14** which is pGEX-2T or pGEX-3X.

17. The plasmid pGEX-1.

18. The plasmid pGEX-2T.

19. The plasmid pGEX-3X.

20. A method of producing a fusion protein comprising glutathione-S-transferase and a second protein or peptide fused directly or indirectly with the COOH-terminus of glutathione-S-transferase which comprises:

(a) transforming a host cell with an expression vector comprising a promoter operatively linked to a nucleotide sequence which codes for a fusion protein wherein said fusion protein comprises glutathione-S-transferase and a second protein or peptide fused directly or indirectly with the COOH-terminus of glutathione-S-transferase and wherein said fusion protein is capable of binding to glutathione;

(b) culturing said host cell under conditions such that said fusion protein is expressed in recoverable quantity;

(c) lysing said host cell; and

(d) purifying said fusion protein by glutathione-affinity chromatography.

21. A method of producing a protein or peptide which comprises:

(a) transforming a host cell with an expression vector comprising a promoter operatively linked to a nucleotide sequence which codes for a fusion protein wherein said fusion protein comprises glutathione-S-transferase and a second protein or peptide fused with the COOH-terminus of glutathione-S-transferase through a cleavable link wherein said fusion protein is capable of binding to glutathione;

(b) culturing said host cell under conditions such that said fusion protein is expressed in recoverable quantity;

(c) lysing said host cell;

(d) purifying said fusion protein by glutathione-affinity chromatography;

(e) cleaving said protein or said peptide from said glutathione-S-transferase; and

(f) isolating said protein or said peptide.

22. A fusion protein comprising glutathione-S-transferase and a second protein or peptide fused directly or indirectly with the COOH-terminus of said glutathione-S-transferase wherein said fusion protein is capable of binding to glutathione.

23. A fusion protein comprising from amino to carboxyl terminus, glutathione-S-transferase, a cleavable linker and a second protein or peptide wherein said fusion protein is capable of binding to glutathione.

24. The fusion protein of claim **23** wherein said cleavable linker is cleavable by a site-specific protease.

25. The fusion protein of claim **24** wherein said protease is selected from the group consisting of thrombin, blood coagulation Factor Xa and renin.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : **5,654,176**

DATED : **August 5, 1997**

INVENTOR(S) : **Donald B. Smith**

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 7, line 3: "ptac11 1" should read –ptac11–

Column 10, line 10: "5'-6terminus" should read –5'-terminus–

Signed and Sealed this

Second Day of March, 1999

Attest:

Attesting Officer

Q. TODD DICKINSON

Acting Commissioner of Patents and Trademarks

EXHIBIT J

EXHIBIT J

United States Patent [19]

Rodwell et al.

[11] **Patent Number: 4,671,958**

[45] **Date of Patent: Jun. 9, 1987**

[54] **ANTIBODY CONJUGATES FOR THE DELIVERY OF COMPOUNDS TO TARGET SITES**

[75] Inventors: **John D. Rodwell**, Yardley; **Thomas J. McKearn**, Narberth, both of Pa.

[73] Assignee: **Cytogen Corporation**, Princeton, N.J.

[21] Appl. No.: **356,315**

[22] Filed: **Mar. 9, 1982**

[51] **Int. Cl.**[4] **A61K 39/00**; A61K 37/00; A23J 37/00

[52] **U.S. Cl.** **424/85**; 514/2; 514/6; 514/8; 530/387; 530/388; 530/389; 530/390; 530/391; 530/828; 424/86; 424/87

[58] **Field of Search** ..... 424/86, 85, 87; 260/112 R; 435/7, 12, 25, 177, 188, 192, 181; 514/2, 6; 530/387, 388, 389, 390, 391, 828

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Primary Examiner—Thomas G. Wiseman
Assistant Examiner—Robin Teskin
Attorney, Agent, or Firm—Pennie & Edmonds

[57] **ABSTRACT**

A method is described for the covalent attachment of linker groups to specific sites on antibody molecules directed against any desired target antigen (tumor, bacterial, fungal, viral, parasitic etc.). These linkers can be attached via amide or ester bonds to compounds for delivery which contain available amino or hydroxy groups (e.g., bioactive agents, cytotoxic agents, dyes, fluors, radioactive compounds, etc.). In addition the linkers can be incorporated into insoluble matrices for use in separation schemes which are based upon antibody-antigen interactions.

The linkers may be designed so that they are susceptible to cleavage by any one of the serum complement enzymes. When prepared according to the methods described herein, the resulting modified antibody molecule retains the ability to bind antigen and to fix serum complement. Thus, when administered to a patient the antibody conjugate binds to its target in vivo. As a result of the subsequent activation of the patient's serum complement, the covalently attached compound will be specifically cleaved at the target site by the proteolytic enzymes of the complement system.

49 Claims, 7 Drawing Figures

6.4.2 HEMOLYTIC ASSAY FOR COMPLEMENT FIXATION

A 200 ul aliquot of a suspension of sheep red blood cells (Gibco Diagnostics, Madison, Wis.) at an approximate concentration of 2×10^8 cells/ml were mixed with 20 ul of the antibody conjugate mixture prepared in Section 6.3 (approximately 2 ug of protein). After 15 minutes of mixing and incubating at 37° C., 100 ul of the human serum complement (prepared in Section 6.4.1) was added to the mixture. After 30 min to 1 hour of incubation at 37° C., the mixture was centrifuged to pellet the cells. The extent of complement-mediated cell lysis was determined by spectrophotometrically measuring hemoglobin released into the supernatant (412 nm).

The results of this assay demonstrated complete hemolysis and essentially 100% binding of antibody to cell surface. For example, addition of distilled water to a pellet formed by centrifuging 200 ul of the sheep red blood cell suspension completely lyses the cells, and releases hemoglobin. A 1:20 dilution of the supernatant of sheep red blood cells which were completely lysed in distilled water had an $O.D._{412}$ of 0.646. An identical dilution of sheep red blood cells which were lysed by the addition of conjugate and complement had an $O.D._{412}$ of 0.672. Thus the conjugate retained the ability to bind antigen and to fix complement.

6.4.3 NON-HEMOLYTIC ASSAY FOR COMPLEMENT MEDIATED RELEASE OF AMC

Conditions for the non-hemolytic assay were identical to those above except that glutaraldehyde-fixed sheep red blood cells (Sigma, St. Louis, Mo.) were used in place of normal sheep red blood cells. Glutaraldehyde fixed cells do not lyse in the presence of antibody and complement and, therefore, no hemoglobin is released. Instead, a fluorometric assay is used to demonstrate the release of the AMC. A non-hemolytic system is necessary for use in the fluorometric assay, because the presence of hemoglobin interferes with fluorescence measurements in this system. Prior to use in the assay, these fixed red blood cells were shown to bind both the unmodified antibody and the Antibody-Phenylhydrazine-Tripeptide-AMC which was prepared in Section 6.3.

The non-hemolytic assay was used to show the specific complement-mediated release of the AMC from the antibody conjugate. Similarly to the hemolytic assay, 200 ul of the glutaraldehyde-fixed sheep red blood cells, at an approximate concentration of 2×10^8 cells/ml, was incubated with the Antibody-Phenylhydrazide-Tripeptide-AMC conjugate at 37° C. for 15 minutes.

After centrifuging and resuspension in buffer, 50 ul of the human complement preparation (Section 6.4.1) was added, and the fluorescense at 460 nm monitored, with excitation at 380 nm (Caporale, et al., 1981, J. Immunol. 126 1963–65.) as a function of time. As controls, the conjugate was incubated with sheep red blood cells alone; in the presence of rat red blood cells and human complement (the monoclonal antibody used does not bind to rat red blood cells); and in the absence of both sheep red blood cells and complement (the monoclonal antibody used does not bind to rat red blood cells). FIG. 7 shows the results of these experiments. A comparision of curve (a) which represents the conjugate incubated with glutaraldehyde-fixed sheep red blood cells and human complement to the control curves labeled (b), (c) and (d) clearly demonstrates the release of free AMC in the sample containing the specific antibody target and human complement. Thus, curve (b) which represents the conjugate incubated with glutaraldehyde-fixed rat red blood cells and human complement, curve (c) which represents the conjugate incubated with glutaraldehyde-fixed sheep red blood cells, and curve (d) which represents the conjugate alone demonstrate no release of AMC.

We claim:

1. A method for in vivo delivery of a compound at an antigenic site, comprising: administering to an individual an effective amount of a soluble antibody conjugate comprising an antibody or antibody fragment directed against said antigenic site and attached to a non-cleavable linker by a covalent bond to an oxidized carbohydrate moiety of the Fc region of the antibody or antibody fragment and in which such linker is covalently attached or complexed to a compound, the soluble antibody conjugate being characterized by (a) substantially the same immuno-specificity as the unconjugated antibody or antibody fragment, and (b) aqueous solubility such that the antibody conjugate is suitable for in vivo administration.

2. The method according to claim 1 wherein the covalent bond is an imine, enamine, hydrazone, oxime, phenylhydrazone, semicarbozone or reduced forms thereof.

3. The method according to claim 1, wherein the antibody fragment is selected from the group consisting of Fab fragments, $(Fab')_2$ fragments and single heavy-light chain pairs.

4. The method according to claim 1, wherein the antibody or antibody fragment is a monoclonal antibody or monoclonal antibody fragment.

5. The method according to claim 1 wherein the antigenic site is a bacterial antigen and the compound is an antibacterial agent.

6. The method according to claim 1, wherin the antigenic site is a viral antigen and the compound is an antiviral agent.

7. The method according to claim 1 wherien the antigenic site is a tumor antigen and the compound is an antitumor agent.

8. The method according to claim 7, wherein the antitumor agent is fluorouracil.

9. The method according to claim 7, wherein the antitumor agent is bleomycin.

10. The method according to claim 7, wherein the antitumor agent is methotrexate.

11. The method according to claim 7, wherein the antitumor agent is adriamycin.

12. The method according to claim 7, wherein the antitumor agent is cerubidine.

13. The method according to claim 7, wherein the antitumor agent is valban.

14. The method according to claim 7, wherein the antitumor agent is alkeran.

15. The method according to claim 1, wherein the antigenic site is a fungal antigen and the compound is an antifungal agent.

16. The method according to claim 1 wherein the antigenic site is a parasite antigen and the compound is an antiparasitic agent.

17. The method according to claim 1 wherein the antigenic site is a mycoplasmal antigen and the compound is an antimycoplasmal agent.

18. The method according to claim 1 wherein the antigenic site is a differentiation or histocompatability antigen and the compound is a cytotoxic agent.

19. The method according to claim 1 wherein the compound is a radiopharmaceutical or a heavy metal.

20. The method according to claim 19 wherein heavy metal is platinum.

21. The method according to claim 1, wherein the compound is a toxin or a toxin fragment.

22. The method according to claim 1, wherein the compound is a neurotransmitter or hormone.

23. The method of claim 1 wherein the compound is an enzyme or a DNA sequence.

24. A method for in vivo delivery and release of a compound at an antigenic site, comprising: administering to an individual an effective amount of a soluble antibody conjugate comprising an antibody or antibody fragment directed against said antigenic site and attached to a cleavable linker by a covalent bond to an oxidized carbohydrate moiety in the Fc region of the antibody or antibody fragment and in which such linker is covalently attached to a compound, the soluble antibody conjugate being characterized by (a) substantially the same immunospecificity as the unconjugate antibody or antibody fragment, (b) aqueous solubility such that the antibody conjugate is suitable for in vivo administration and (c) the linker which is unstable by reason of its ability to be cleaved by activated serum complement or a serum protease.

25. The method according to claim 24 wherein the covalent bond is an imine, enamine, hydrazone, oxime, phenylhydrazone, semicarbazone, or reduced forms thereof.

26. The method according to claim 24 wherein the antibody fragment is selected from the group consisting of Fab fragments, $(Fab')_2$ fragments and single heavy-light chain pairs.

27. The method according to claim 24 wherein the antibody or antibody fragment is a monoclonal antibody or monoclonal antibody fragment.

28. The method according to claim 24 wherein the linker is cleavable by activated C1.

29. The method according to claim 24 wherein the linker is cleavable by activated C4, 2.

30. The method according to claim 24 wherein the linker is cleavable by a serum protease.

31. The method according to claim 24 wherein the antigenic site is a bacterial antigen and the compound is an antibacterial agent.

32. The method according to claim 24 wherein the antigenic site is a viral antigen and the compound is an antiviral agent.

33. The method according to claim 24 wherein the antigenic site is a tumor antigen and the compound is an antitumor agent.

34. The method according to claim 33 wherein the antitumor agent is fluorouracil.

35. The method according to claim 33 wherein the antitumor agent is bleomycin.

36. The method according to claim 33 wherein the antitumor agent is methotrexate.

37. The method according to claim 33 wherein the antitumor agent is adraimycin.

38. The method according to claim 33 wherein the antitumor agent is cerubidine.

39. The method according to claim 33 wherein the antitumor agent is valban.

40. The method according to claim 33 wherein the antitumor agent is alkeran.

41. The method according to claim 24 wherein the antigenic site is a fungal antigen and the compound is an antifungal agent.

42. The method according to claim 24 wherein the antigenic site is a parasitic antigen and the compound is an antiparasitic agent.

43. The method according to claim 24 wherein the antigenic site is a mycoplasmal antigen and the compound is an antimycoplasmal agent.

44. The method according to claim 24 wherein the antigenic site is a differentiation or histocompatability antigen and the compound is an cytotoxic agent.

45. The method according to claim 24 wherein the compound is an radiopharmaceutical or a heavy metal.

46. The method according to claim 24 wherein the compound is an toxin or a toxin fragment.

47. The method according to claim 24 wherein the compound is a neurotransmitter or hormone.

48. The method of claim 24 wherein the compound is an enzyme or a DNA sequence.

49. A method for in vivo delivery of a compound at an antigenic site, comprising: administering to an individual an effective amount of a soluble antibody conjugate comprising an antibody or antibody fragment directed against the antigenic site and attached to a cleavable or non-cleavable liner by a covalent bond of an oxidized carbohydrate moiety of the Fc region of the antibody or of the antibody fragment and in which such linker is covalently attached or complexed to a compound, the soluble antibody conjugate being characterized by (a) substatially the same immunospecificity as the unconjugated antibody or antibody fragment, and (b) aqueous solubility such that the antibody conjugate is suitable for in vivo administration.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,671,958

DATED : June 9, 1987

INVENTOR(S) : John D. Rodwell, Thomas J. McKearn

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, References cited

On page 1, correct the Karush publication by deleting "Anti-Lactive" and inserting --Anti-Lactose--; by deleting page numbers "2226-2231" and inserting --2226-2232--.

On page 1, correct the Hurwitz publication by deleting "Dainorubicin" and inserting -Daunorubicin--.

On page 1, correct the Monsigny publication by deleting "Drug-Carrier-Counter (PAC Conjugate)" and inserting --Drug-And-Carrier (DAC Conjugate)--.

On page 2, correct the Hobart publication by deleting "on,the Molecular Cellular" and inserting --on the Molecular and Cellular--.

On page 2, correct the date of the Leatherbarrow publication by deleting "1983" and inserting --1985--.

Column 4, line 23 insert a "," (comma) between "fungi" and "bacteria".

Table 1 delete "Valban" and insert --Velban--.

Column 9, line 24 delete "has an hydroxy" and insert -- has a hydroxy--.

Column 21, line 59 delete "126 1963-65)" and insert --126:1963-65)--.

UNITED STATES PATENT AND TRADEMARK OFFICE

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PATENT NO. : 4,671,958

DATED : June 9, 1987

INVENTOR(S) : John D. Rodwell, Thomas J. McKearn

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 22, line 28 delete "semicarbozone" and insert --semicarbazone--.

Column 22, line 40 delete "wherin" and insert --wherein--.

Column 22, line 43 delete "wherien" and insert --wherein--.

Column 22, line 57 delete "valban" and insert --velban--.

Column 23, line 24 delete "unconjugate" and insert --unconjugated--.

Column 24, line 11 delete "adraimycin" and insert --adriamycin--.

Column 24, line 15 delete "valban" and insert --velban--.

Column 24, line 29 delete "an" and insert --a--.

Column 24, line 31 delete "an" and insert --a--.

Column 24, line 33 delete "an" and insert --a--.

Column 24, line 43, delete "liner" and insert --linker--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,671,958

DATED : June 9, 1987

INVENTOR(S) : John D. Rodwell, Thomas J. McKearn

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 24, line 48 delete "substatially" and insert --substantially--.

Signed and Sealed this

Twelfth Day of April, 1988

Attest:

DONALD J. QUIGG

Attesting Officer

Commissioner of Patents and Trademarks